Ca²⁺-induced accumulation of pyrophosphate in mitochondria during acetate metabolism

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The mechanism of pyrophosphate (PP_i) accumulation in rat liver during acetate metabolism was investigated. Perfusion of the liver with acetate in the presence of noradrenaline and glucagon induced marked accumulation of PP_i (2 μmol/g of liver, 200 times that of control). In contrast, perfusion with glutamine, which generates PP_i only in the cytosol, caused little accumulation of PP_i, even in the presence of the two hormones. The site of PP_i accumulation was shown to be the mitochondria by the finding that isolated mitochondria from the liver perfused with acetate and the hormones contained 50 nmol of PP_i/mg of protein. The addition of an uncoupler to mitochondria with accumulated PP_i caused gradual decrease in their PP_i content, with concomitant release of a stoichiometric amount of Ca²⁺. Similar accumulation of PP_i was observed when isolated mitochondria were incubated with acetate and Ca²⁺. These results show that an increase in cytosolic Ca²⁺ caused by the co-administration of the two hormones induced uptake of the ion into mitochondria, and that PP_i accumulated in mitochondria only when it was generated in the organelles with an elevated concentration of Ca²⁺. High mitochondrial concentrations of Ca²⁺ are considered to inhibit inorganic pyrophosphatase through the formation of a stable complex, CaPP_i⁻. Mitochondria with accumulated PP_i had normal respiratory activities, and their adenine nucleotide concentrations were increased 2-fold rather than being decreased, the increases also being considered to be caused by their high concentration of Ca²⁺.

INTRODUCTION

In mammalian tissues, acetate is converted into acetyl-CoA by acetyl-CoA synthetase (EC 6.2.1.1) by the following reaction, generating PP_i:

Intraperitoneal injection of acetate into starved rats has been reported to increase the PP_i concentration in the liver to more than 100 times that in normal liver (Veech *et al.*, 1986; Veech & Gitomer, 1988). In a previous paper we reported that a high intracellular level of Ca²⁺ caused by the synergistic actions of Ca²⁺-mobilizing hormones, such as noradrenaline, and glucagon is indispensable for the accumulation of PP_i in perfused rat liver (Yamada *et al.*, 1988). This observation is consistent with the findings that the accumulation of PP_i was much less in fed animals (Veech *et al.*, 1980, 1986; Veech & Gitomer, 1988; T. Inoue, unpublished work), and that isolated hepatocytes accumulated PP_i in the presence of a Ca²⁺ ionophore (Gitomer & Veech, 1986). Otto & Cook (1982) and Davidson & Halestrap (1987) reported a Ca²⁺-induced increase in PP_i in isolated mitochondria. Moreover, Davidson & Halestrap (1988) have demonstrated the compartmentation of PP_i within the isolated hepatocytes, using a rapid fractionation technique. They have indicated that PP_i is almost entirely intramitochondrial, and that treatment with glucagon and vasopressin increases the PP_i concentration to 4 times that in control cells. In acetate metabolism, PP_i is formed in both the cytosol and the mitochondria (Aas & Bremer,

1968; Barth *et al.*, 1971), but Gitomer & Veech (1986) and we (Yamada *et al.*, 1988) also suggested that PP_i accumulated mainly in mitochondria. Under physiological conditions, mitochondria are not regarded as very important organelles in the regulation of cytosolic Ca²⁺ (Somlyo *et al.*, 1985; Carafoli, 1987), because of their low-affinity uptake system for the ion. However, they are considered to buffer a large amount of excess Ca²⁺ when its cytosolic concentration increases pathologically to levels corresponding to their affinity (Rasmussen & Barrett, 1984; Carafoli, 1987). As in pathological conditions, the co-administration of a Ca²⁺-mobilizing hormone and glucagon is reported to lead to synergistic potentiation of Ca²⁺ influx in liver, the magnitude of which is much greater than that with a Ca²⁺-mobilizing hormone alone (Morgan *et al.*, 1983; Mauger *et al.*, 1985; Altin & Bygrave, 1986). In fact, accumulation of Ca²⁺ in mitochondria after hormonal treatment was observed by cell-fractionation studies (Morgan *et al.*, 1983; Altin & Bygrave, 1986). Noradrenaline and glucagon have also been reported to increase the adenine nucleotide content of liver mitochondria (Siess *et al.*, 1977; Titheradge & Haynes, 1980; Aprille *et al.*, 1982; Soboll & Scholz, 1986), a phenomenon that is also considered to be associated with the transfer of intracellular Ca²⁺ from the cytosol to mitochondria (Haynes *et al.*, 1986; Davidson & Halestrap, 1987). But if PP_i and Ca²⁺ accumulate in mitochondria, the ATP level can be decreased as a result of mitochondrial dysfunction.

In the present study, we isolated mitochondria from

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazine.

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perfused rat liver to examine hormonal effects on the mitochondrial contents of PP_i and adenine nucleotides. Our results show that mitochondria are the site of PP_i accumulation, and that a high intracellular and, subsequently, mitochondrial Ca^{2+} concentration is responsible for this PP_i accumulation. In spite of marked accumulation of PP_i , the mitochondria showed no deterioration of function.

MATERIALS AND METHODS

Liver perfusion and preparation of mitochondria

Male Sprague-Dawley rats weighing 200–250 g were starved for 24 h before use. Liver perfusion was carried out as described by Sugano *et al.* (1978) with some modifications (Yamada *et al.*, 1988). Mitochondria were isolated from perfused liver and liver *in situ* in 0.3 M-mannitol/10 mM-Hepes/KOH/0.2 mM-EDTA/0.1 % bovine serum albumin, pH 7.4 (Watanabe *et al.*, 1983). They were washed twice with, and then suspended in, this medium, to which 4 mM- K_2HPO_4 /1 mM- KH_2PO_4 , pH 7.4, was added instead of 0.2 mM-EDTA (suspension medium). The whole isolation procedure was performed within 1 h, and all experiments were completed within 2 h after isolation of mitochondria.

Analytical methods

Respiratory activities of mitochondria were determined with an oxygen electrode (PC-100; Sumitomo Electric Industries, Osaka, Japan) at 25 °C with succinate as a substrate. Samples for assays of PP_i , adenine nucleotides and acetate were prepared from perfused livers (Yamada *et al.*, 1988) and from mitochondrial suspensions (Watanabe *et al.*, 1985) by extracting the preparations with 0.5 M- $HClO_4$. For measurement of the intramitochondrial PP_i and adenine nucleotides, mitochondria were separated from the external medium by the centrifuge-column method (Watanabe *et al.*, 1983). PP_i was determined by the method of Cook *et al.* (1978). Adenine nucleotides were determined by h.p.l.c. on a reverse-phase column of Shim-pack CLC-ODS (6 mm × 150 mm; Shimadzu Co., Kyoto, Japan) equilibrated with 88 mM- NaH_2PO_4 /12 mM- Na_2HPO_4 ,

pH 6.0, and 0.8 % (v/v) methanol. Acetate was determined by h.p.l.c. on a reverse-phase column of YMC-pack AQ-312 (6 mm × 150 mm; Yamamura Chemical Laboratories Co., Kyoto, Japan) equilibrated with 0.1 M- H_3PO_4 /NaOH, pH 2.8, and 3 % (v/v) methanol. Release of Ca^{2+} from mitochondria was measured with Arsenazo III as described previously (Tagawa *et al.*, 1985). Urea was measured as described by Kerscher & Ziegenhorn (1985). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Materials

Chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A., and Wako Pure Chemical Industries, Osaka, Japan. Enzymes were from Boehringer, Mannheim, Germany, or Sigma.

RESULTS

Effects of hormones, acetate and glutamine on PP_i and adenine nucleotide concentrations in perfused rat liver

In our previous study (Yamada *et al.*, 1988), the addition of acetate alone or with a single hormone to the perfusion medium increased the PP_i concentration in the liver to up to 10 times the control value. This increase was much less than that observed *in vivo* after intraperitoneal injection of acetate. Acetate, glucagon and noradrenaline were all required for accumulation of PP_i to a similar level to that *in vivo*. Table 1 shows these results together with the hepatic concentrations of adenine nucleotides under these conditions, measured because the intracellular concentration of ATP is a good index of the severity of cell injury caused by mitochondrial dysfunction. No marked change in adenine nucleotide concentrations was observed, irrespective of accumulation of PP_i . The addition of acetate in the presence and absence of the hormones slightly decreased the concentration of ATP and increased those of ADP and AMP inversely, but this decrease scarcely affected the physiological functions of the liver (Kamiike *et al.*, 1985; Nishida *et al.*, 1987).

Glutamine activates urea synthesis when it is added to

Table 1. PP_i and adenine nucleotide concentrations in perfused rat liver

Livers were perfused for 30 min at 30 °C with various compounds at the following concentrations: acetate, 10 mM; noradrenaline, 10 μ M where not indicated otherwise; glucagon, 10 nM; glutamine, 10 mM. Then the livers were freeze-clamped and their concentrations of PP_i and adenine nucleotides were measured. A high concentration of noradrenaline was used, on the basis of previous results (Yamada *et al.*, 1988): 100 nM-noradrenaline gave similar results. Values are means $\pm$ S.D. for *n* independent experiments.

Additions	<i>n</i>	Concn. (μ mol/g of liver)				
		PP_i	ATP	ADP	AMP	ATP + ADP + AMP
None	6	0.019 $\pm$ 0.006	2.55 $\pm$ 0.24	0.76 $\pm$ 0.20	0.21 $\pm$ 0.11	3.51 $\pm$ 0.44
Acetate	3	0.086 $\pm$ 0.014				
Acetate + noradrenaline	4	0.22 $\pm$ 0.11				
Acetate + glucagon	5	0.24 $\pm$ 0.05	2.02 $\pm$ 0.52	0.98 $\pm$ 0.29	0.73 $\pm$ 0.18	3.73 $\pm$ 0.25
Acetate + noradrenaline + glucagon	6	2.03 $\pm$ 0.57	1.97 $\pm$ 0.27	1.21 $\pm$ 0.18	0.71 $\pm$ 0.18	3.89 $\pm$ 0.35
Acetate + 1 μ M-noradrenaline + glucagon	6	2.02 $\pm$ 0.53	2.02 $\pm$ 0.14	1.37 $\pm$ 0.25	0.64 $\pm$ 0.15	3.99 $\pm$ 0.41
Noradrenaline + glucagon	7	0.038 $\pm$ 0.011	2.23 $\pm$ 0.43	1.01 $\pm$ 0.26	0.28 $\pm$ 0.16	3.52 $\pm$ 0.49
Glutamine + noradrenaline + glucagon	4	0.032 $\pm$ 0.006

Fig. 1. Activation of urea synthesis by glutamine in perfused rat liver

Liver was perfused with 1 μ M-noradrenaline, 10 nM-glucagon, and glutamine at the indicated concentrations. Urea in the effluent (○) was determined as described in the Materials and methods section. After perfusion for 30 min, PP_i contents (●) were measured. Values are means $\pm$ S.D. for at least three experiments.

the perfusion fluid of rat liver as a nitrogen source. Fig. 1 shows that urea output from the liver was fully accelerated with more than 10 mM-glutamine in the perfusate. Since urea synthesis is one of the most active PP_i-generating systems in rat liver, we examined the effect of glutamine instead of acetate on PP_i accumulation. As shown in Fig. 1, no accumulation of PP_i was observed even in the presence of 10 mM- or 20 mM-glutamine. The rates of ureogenesis in these conditions were more than 0.9 μ mol/min per g of liver (Fig. 1), which was more than the rate of ketogenesis induced by acetate (0.3 μ mol/min per g of liver; Yamada *et al.*, 1988). Thus as much PP_i can be generated as in the case of acetate metabolism. In urea synthesis, PP_i is formed only in the cytosol, and these results therefore indicate that PP_i accumulates in a liver only when it is formed in mitochondria.

Accumulations of PP_i, adenine nucleotides and Ca²⁺ in mitochondria isolated from perfused liver

To examine the mitochondrial accumulation of PP_i, we isolated mitochondria from perfused liver. As shown

in Table 2, perfusion with acetate in the presence of noradrenaline and glucagon induced a marked increase in the mitochondrial concentration of PP_i, whereas perfusion with acetate or these hormones alone caused scarcely any increase in mitochondrial PP_i. These findings were consistent with the results obtained with whole liver (Table 1), but the magnitude of accumulation, 50 times that of the control, was somewhat lower than that observed in whole liver. In spite of the unphysiological accumulation of PP_i, these mitochondria seemed to be intact, because their respiratory control ratio was unaffected (Table 2).

Another remarkable effect of the hormones on mitochondria was their induction of accumulation of adenine nucleotides. Table 2 shows that the administrations of glucagon and noradrenaline with or without acetate increased the levels of intramitochondrial adenine nucleotides, mainly ATP, to twice the control values. The concentrations of adenine nucleotides in whole liver did not increase under these conditions (Table 1), and hence this increase in mitochondrial ATP was the result of its subcellular redistribution.

As the accumulations of both PP_i and adenine nucleotides are considered to be associated with mitochondrial uptake of Ca²⁺ (Gitomer & Veech, 1986; Soboll & Scholz, 1986; Haynes *et al.*, 1986; Davidson & Halestrap, 1987, 1988; Yamada *et al.*, 1988), we examined the release of Ca²⁺ from mitochondria with accumulated PP_i. CCCP is an uncoupling agent that causes rapid hydrolysis of ATP and concomitant release of Ca²⁺ from isolated mitochondria (Tagawa *et al.*, 1985). Fig. 2(a) shows the time course of the uncoupler-induced Ca²⁺ release from mitochondria isolated from perfused liver before and after treatment with acetate and hormones. Almost all of the exchangeable Ca²⁺ was released within 1 min from control mitochondria, but release of Ca²⁺ from the mitochondria of the same liver after treatment with acetate and hormones continued until 30 min after the addition of CCCP. Moreover, the total amount of Ca²⁺ released from the latter was over 25 nmol/mg of protein, which was much more than that from control mitochondria. As shown in Fig. 2(b), the PP_i content of mitochondria decreased slowly, while ATP was rapidly hydrolysed. Of the total amount of PP_i that decreased, a small portion was recovered in the external medium (results not shown), the remainder being degraded. This decrease in PP_i content seemed to be a mirror image of the difference in Ca²⁺ release from mitochondria with accumulated PP_i and control mitochondria shown in Fig. 2(a): the decreases in PP_i content at 1, 10 and 30 min

Table 2. PP_i and adenine nucleotide concentrations in mitochondria isolated from perfused liver

Mitochondria were isolated from livers perfused with or without 10 mM-acetate, 1 μ M-noradrenaline and 10 nM-glucagon for 30 min. Immediately after their isolation, their respiratory activities and concentrations of PP_i and adenine nucleotides were measured. Abbreviation: RCR, respiratory control ratio. Values are means $\pm$ S.D. for *n* independent experiments.

Additions	<i>n</i>	Concn. (nmol/mg of protein)					RCR
		PP _i	ATP	ADP	AMP	ATP+ADP+AMP	
None	6	1.01 $\pm$ 0.31	12.1 $\pm$ 3.0	2.9 $\pm$ 1.2	4.7 $\pm$ 3.2	19.7 $\pm$ 3.1	4.3 $\pm$ 0.4
Acetate	3	1.98 $\pm$ 0.58	12.4 $\pm$ 3.3	2.0 $\pm$ 0.2	4.1 $\pm$ 0.8	18.4 $\pm$ 2.3	4.0 $\pm$ 0.1
Acetate + noradrenaline + glucagon	4	50.6 $\pm$ 7.6	20.6 $\pm$ 5.8	7.5 $\pm$ 4.0	6.6 $\pm$ 4.1	34.7 $\pm$ 4.0	4.0 $\pm$ 0.5
Noradrenaline + glucagon	3	5.32 $\pm$ 1.99	25.6 $\pm$ 2.1	8.6 $\pm$ 1.9	6.4 $\pm$ 4.2	40.5 $\pm$ 6.0

Fig. 2. Uncoupler-induced Ca^{2+} release from PP_i -accumulated mitochondria

Mitochondria were isolated from liver perfused with 10 mM-acetate, 1 μM -noradrenaline and 10 nM-glucagon for 30 min, and were suspended in the suspension medium at a protein concentration of 18 mg/ml. CCCP was added at a final concentration of 5 μM , and then the suspension was incubated at 25 °C. (a) Free Ca^{2+} was measured in the supernatant obtained by centrifugation at 8000 g for 40 s at 0 °C (●). ○, Ca^{2+} released from mitochondria isolated from the same liver before treatment. (b) PP_i (●, ○) and ATP (▲, △) concentrations in mitochondria with accumulated PP_i (●, ▲) and control mitochondria (○, △). Broken lines represent results of a control incubation in the absence of CCCP under aerobic conditions. Results are for one of three experiments with similar results.

Fig. 3. Accumulation of PP_i during acetate metabolism in isolated mitochondria

Mitochondria (21 mg of protein/ml) were incubated with the suspension medium containing 2 mM-ATP under aerobic conditions at 25 °C. At the times indicated, the PP_i (a) and acetate (b) concentrations in HClO_4 extracts were measured. Symbols: ●, with 50 nmol of Ca^{2+} /mg of protein; □, with 10 mM-acetate; ■, with both Ca^{2+} and acetate. The results of one of three experiments with similar results are shown.

were 2.1, 12.1 and 22.7 nmol/mg of protein respectively, and the differences in Ca²⁺ release were 5.8, 13.4 and 22.3 nmol/mg of protein respectively. In the absence of an uncoupler, no Ca²⁺ was released and the PP_i content was not decreased (Fig. 2).

Accumulation of PP_i in isolated mitochondria

Otto & Cook (1982) and Davidson & Halestrap (1987, 1988) observed that PP_i accumulated in isolated mitochondria during oxidation of butyrate in the presence of Ca²⁺. To confirm the relationship between accumulation of PP_i and Ca²⁺ in mitochondria, we incubated isolated mitochondria with acetate in the presence and absence of a large amount of Ca²⁺. Fig. 3 shows the accumulation of PP_i in Ca²⁺-loaded mitochondria during acetate metabolism. The accumulation reached a maximum after incubation for 20 min. When PP_i reached this level, acetate was no longer metabolized in isolated mitochondria (Fig. 3b) and synthesis of ketone bodies also ceased (results not shown). The suppression of acetate metabolism in Ca²⁺-loaded mitochondria was considered to be due to product inhibition of acetyl-CoA synthetase by PP_i (Otto & Cook, 1982).

DISCUSSION

PP_i is generated in several metabolic processes such as activation of fatty acids and amino acids, the urea cycle and glycogen synthesis. These reactions mainly occur in the cytosol, but some of them, such as activation of short-chain fatty acids, also occur in mitochondria. All these reactions are driven forward by the hydrolysis of PP_i, because their equilibrium is unfavourable for PP_i formation. Thus, for the accumulation of a large amount of PP_i, it must be trapped by some mechanism. Davidson & Halestrap (1988) have reported that PP_i is almost entirely mitochondrial in control and hormone-stimulated hepatocytes. They have suggested that PP_i accumulates, to result in precipitation of MgPP_i or CaPP_i. In the present study, we confirmed the mitochondrial accumulation in the perfused liver, where as much PP_i was accumulated as in livers *in situ* after intraperitoneal injection of acetate. The results in Fig. 1, where glutamine did not induce any changes in PP_i concentration, strongly suggest this mitochondrial accumulation. Actually, mitochondria isolated from liver in which PP_i was accumulated contained a large amount of PP_i (Table 2). The magnitude of PP_i accumulation in mitochondria was lower than the value obtained by direct extraction of perfused livers (Table 1). This difference can be explained by higher PP_i content of the control mitochondria than that reported by others (Otto & Cook, 1982; Davidson & Halestrap, 1987). The mitochondrial preparation used in the present study showed a small incorporation of Ca²⁺ (2 nmol/mg of protein) from the washing medium without chelating agents. This may lead to a small (< 0.5 nmol/mg of protein) increase in the concentration of PP_i in the mitochondria obtained. In fact, Davidson & Halestrap (1987) have indicated that matrix PP_i increases with sub-micromolar concentrations of extramitochondrial Ca²⁺.

Mitochondrial accumulation of PP_i was also shown in the experiment with isolated mitochondria in the presence of acetate and Ca²⁺ (Fig. 3). The requirement of Ca²⁺ for the accumulation of PP_i is consistent with the facts that Ca²⁺-mobilizing hormones are essential for PP_i accumu-

lation in perfused liver, and that omission of Ca²⁺ from the perfusion medium or addition of a Ca²⁺ antagonist decreased PP_i accumulation (Yamada *et al.*, 1988). The co-administration of a Ca²⁺-mobilizing hormone and glucagon induces much larger influx of Ca²⁺ across the plasma membrane of liver than that with a Ca²⁺-mobilizing hormone alone, although the mechanism of this effect is unknown (Morgan *et al.*, 1983; Mauger *et al.*, 1985; Altin & Bygrave, 1986). When the environmental level of Ca²⁺ is elevated, mitochondria can rapidly take up large amounts of the ion (there are many reports, reviewed by Carafoli, 1987). Ca²⁺ has been reported to be a potent inhibitor of pyrophosphatase (Irie *et al.*, 1970), and Otto & Cook (1982) reported that Ca²⁺ also decreases the availability of PP_i for the enzyme by forming calcium pyrophosphate. We have found that addition of PP_i to a solution of Ca²⁺ decreased the activity of the ion through formation of a CaPP_i⁻ complex. The results in Fig. 2, where the release of Ca²⁺ was parallel to the decrease in intramitochondrial PP_i, suggest dissociation of the complex formed in the matrix. A collapse of membrane potential induced Ca²⁺ efflux, and resulted in a decrease in free Ca²⁺ concentration of the matrix. This decrease in the matrix Ca²⁺ activity was considered to dissociate the complex and to liberate free PP_i, which was degraded or released into the medium. From these results, we conclude that PP_i accumulated in the form of a complex in living cells.

In isolated mitochondria, the consumption of acetate and the accumulation of PP_i ceased after 20 min when Ca²⁺ was present (Fig. 3). However, in perfused liver, the consumption of acetate and the formation of ketone bodies were sustained for several hours (Yamada *et al.*, 1988). Acetate can also be metabolized by an active cytosolic acetyl-CoA synthetase, and then the acetyl-CoA generated may enter the mitochondria as acetylcarnitine (Bieber, 1988).

Increases in the concentrations of adenine nucleotides have been observed in mitochondria isolated from glucagon-treated rats and hepatocytes (Titheradge & Haynes, 1980; Aprille *et al.*, 1982) and from the liver perfused with glucagon or adrenaline (Soboll & Scholz, 1986). However, in these experiments, the hormones were administered singly, and caused less increase than in the present study. Davidson & Halestrap (1987) proposed the following mechanism for the increase: a rise in cytosolic concentration of Ca²⁺ caused by hormones induces uptake of Ca²⁺ into mitochondria. This then increases the mitochondrial concentration of PP_i, in exchange for which cytosolic adenine nucleotides are taken up via adenine nucleotide translocase. On the other hand, Haynes *et al.* (1986) reported that neither carboxyatractyloside nor Ruthenium Red affected the Ca²⁺-dependent uptake of ATP into isolated mitochondria, and that the uptake was enhanced under a diminished membrane potential. They therefore suggested the net uptake of ATP as a complex with Ca²⁺ in a translocase-independent manner. In the present study, perfusion with acetate alone did not cause any changes in nucleotide content, but doubled that of PP_i (Table 2). Noradrenaline and glucagon increased the nucleotide content in the presence and absence of acetate (Table 2). When CCCP was added, a large amount of Ca²⁺ was released not only from mitochondria with accumulated PP_i (Fig. 2a) but also from mitochondria isolated from liver perfused with the hormones alone

(results not shown). A high mitochondrial concentration of Ca^{2+} therefore appeared to be involved in the uptake of adenine nucleotides, but we do not know whether intramitochondrial PP_i is involved. The physiological significance of the increase in the adenine nucleotide pool in mitochondria by hormonal action on the liver is controversial, but a high content of ATP is beneficial to maintain the matrix level of Ca^{2+} (Tagawa *et al.*, 1985), whose release into the cytosol is highly toxic to the cells.

As described previously (Yamada *et al.*, 1988), PP_i could accumulate in human liver, because the concentration of acetate in human plasma is reported to increase to sufficient levels for its accumulation during haemodialysis therapy using medium containing acetate (Desch *et al.*, 1978) and during ethanol intake (Lundquist *et al.*, 1962). Furthermore, some mitochondrial dysfunctions are reported in ethanol-induced liver injury (Lieber, 1984; Mitchell & Herlong, 1986). Although we could not detect any damage to mitochondria with accumulated PP_i in the present study, further investigations may clarify the relation between PP_i accumulation and these dysfunctions.

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